Manganese Superoxide Dismutase Modulates Hypoxia-Inducible Factor-1α Induction via Superoxide

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that plays an important role in O2 homeostasis. Numerous observations suggest that changes in reactive oxygen species affect HIF-1α stabilization and HIF-1α transcriptional activation in many cell types. The antioxidant enzyme manganese superoxide dismutase (MnSOD) modulates the cellular redox environment by converting superoxide (O2−) to hydrogen peroxide and dioxygen. Previous results from our group have shown that overexpression of MnSOD in MCF-7 cells alters stabilization of HIF-1α under hypoxic conditions; however, the underlying mechanism(s) is not known. Here, we tested the hypothesis that MnSOD regulates the expression of HIF-1α by modulating the steady-state level of O2−. We found that decreasing MnSOD with small interfering RNA in MCF-7 cells resulted in (a) an associated increase in the hypoxic accumulation of HIF-1α immunoreactive protein, (b) a significant increase in the levels of O2− (P < 0.01), but (c) no significant change in the steady-state level of O2−. Removal of O2− using spin traps (α-,pyridyl-1-oxide-N-tert-butyl-nitrone and 5,5-dimethyl-1-pyrroline N-oxide) or the O2− scavenger Tempol or an SOD mimic (AEOL10113) resulted in a decrease in HIF-1α protein, consistent with the hypothesis that O2− is an important molecular effector responsible for hypoxic stabilization of HIF-1α. The evidence from both genetic and pharmaceutical manipulation is consistent with our hypothesis that O2− can contribute to the stabilization of HIF-1α.

Introduction

The transcription factor hypoxia-inducible factor (HIF) is a key regulator of the cellular response to O2 homeostasis. HIF up-regulates the expression of many genes, including those responsible for angiogenesis, glycolysis, cell growth, cell survival, and metastasis (1, 2). HIF is a heterodimer composed of a constitutively expressed β subunit and an oxygen-regulated α subunit (3, 4). There are three known forms of HIF: HIF-1, HIF-2, and HIF-3. The immediate response to hypoxia is principally mediated through an increase in the level of HIF-1α, a ubiquitously expressed protein in most cell types. When O2 is adequate, two prolyl residues at the NH2 terminal activation domain of HIF-1α are targeted for hydroxylation by appropriate prolyl hydroxylase domain-containing proteins (PHD). Upon hydroxylation, HIF-1α binds to the Von Hippel-Lindau (pVHL) tumor suppressor protein and leads to its ubiquitination and subsequent degradation via the 26s proteasome (5–7). When there is inadequate O2 in the cell for this hydroxylation reaction, HIF-1α does not bind to pVHL; thus, it accumulates and translocates to the nucleus where it dimerizes with HIF-1β, leading to formation of the transcription factor HIF. HIF will bind to hypoxia-responsive elements within genes initiating their expression, for example, vascular endothelial growth factor (VEGF) and erythropoietin (8).

Manganese superoxide dismutase (MnSOD) is a primary antioxidant enzyme (AE) that localizes in the mitochondrial matrix of eukaryotic cells. MnSOD is essential for maintaining normal tissue function. It modulates the intracellular redox environment by dismutating O2− produced by the electron transfer chain in mitochondria forming H2O2 and O2: O2− + O2− → 2H+ → H2O2 + O2. The majority of tumors have greater steady-state levels of O2− due to loss of MnSOD (9). Multiple studies have shown that reactive oxygen species (ROS) generated from mitochondria can participate in the hypoxia signal transduction pathway that mediates HIF-1α stabilization (10–12). Lower levels of MnSOD protein and its activity have been found in many types of tumors (13, 14), and one of such sample is MCF-7 cells (15). Moderate overexpression of MnSOD in MCF-7 cells has been shown to suppress hypoxic accumulation of HIF-1α protein at both 1% and 4% O2 (16). The downstream effects of HIF-1α suppression by elevated levels of MnSOD activity resulted in a decrease in the secretion of VEGF protein in cells exposed to 1% O2 (16). Alternatively, overexpressing MnSOD or CuZnSOD in A549 human lung epithelial cells does not alter HIF-1α stabilization under hypoxic conditions, whereas overexpressing GPx1 or catalase decreased HIF-1α accumulation at low O2 levels (17). In both of these latter studies, changes in the levels of ROS were not reported.

Although the effects of MnSOD on HIF-1α stabilization have been reported, the mechanism underlying MnSOD-mediated HIF-1α regulation and the effect of ROS removal on HIF-1α in response to hypoxia have not been clearly defined. We hypothesize that MnSOD affects the expression of redox-sensitive genes, including HIF-1α, by modulating ROS levels in cells. We used molecular genetic and chemical approaches for ROS manipulation to analyze the regulation of HIF-1α during hypoxia. We observed that decreasing the level of MnSOD by small interfering RNA (siRNA) transfection elevated the levels of O2− and induced the accumulation of HIF-1α. This induction of HIF-1α was suppressed when O2− was removed using O2− scavengers or an SOD mimic. Here, we propose that MnSOD plays an important role in regulating the accumulation of HIF-1α during hypoxia by modulating the level of O2−.

Materials and Methods

Reagents and chemicals. AEOL10113 [manganese (III) meso-tetrakis (N-ethylpyridinium-2-yl) porphyrin] was a gift from Dr. James D. Crapo of...
National Jewish Medical Research Center. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was from Dojindo. Para-hydroxy phenyl acetic acid (pHPA), horseradish peroxidase (HRP), α-4-pyridyl-1-oxide-N-tert-butyl nitronite (POBN), and 4-hydroxy-2,2,6,6-tetramethylpiperidinoloxyl (Tempol) were from Sigma.

Cell culture. Human immortalized nonmalignant mammary epithelial cells, MCF10A, were cultured in DMEM/Ham's F-12 (1:1) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin, and 500 ng/mL hydrocortisone. Human breast adenocarcinoma MCF-7 cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 0.1 mmol/L nonessential amino acids. Cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO₂.

Induction of hypoxia. Cells were seeded into 60-mm culture dishes; fresh hypoxia medium was provided before hypoxic or chemical treatments. For hypoxia experiments, the dishes were placed in a hypoxic chamber (Billups-Rothenberg) and flushed with 1% O₂ (premixed 1% O₂, 5% CO₂, 94% N₂) for 5 min at 20 L/min, then the gas exchange ports were closed and the chamber was placed in an incubator at 37°C for 4 h.

Inhibition of MnSOD by RNA interference. The predesigned double-stranded siRNA and its complement directed against MnSOD (5'-GGGCC-UAGAUUAUCUAAAAGGCTT-3') and the nonspecific siRNA, the commercially available nontargeting siRNA and its complement were purchased from Ambion, Inc. Briefly, 1 x 10⁶ cells were seeded into 60-mm dishes the day before transfection. After 24 h, the media was replaced with OptiMEM (Life Technologies). Cells were then transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. After 24 h, the transfection media was replaced with regular complete media without antibiotics. After 72 h, cells were harvested or treated with hypoxia for further experiments.

Protein harvest for HIF-1α. Medium was removed from tissue culture dishes, and cells were rinsed twice with cold PBS and then aspirated. Boiling lysis buffer [1% SDS, 1 mmol/L sodium ortho- vanadate, and 10 mmol/L Tris buffer (pH 7.4)] was added to the cells (16). Cells were quickly scraped and transferred into microcentrifuge tubes and boiled for 5 min. The viscosity was reduced by passing the lysates through a 25-gauge needle and then centrifuging at 12,000 x g, 4°C for 10 min, and the supernatants were transferred to a new tube. Protein concentration was determined with Bio-Rad detergent-compatible protein assay.

Western blot analysis. Analysis of HIF-1α protein used 4% to 20% gradient Tris-HCl polyacrylamide ready-to-use gels (Bio-Rad) and electrotransferred onto a polyvinylidene difluoride membrane. Mouse monoclonal antibody to HIF-1α (PharMingen/Transduction Laboratories) was used as a primary antibody, whereas mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion) was used as a primary antibody for loading control protein. Goat anti-mouse IgG (PharMingen/Transduction Laboratories) was used as a secondary antibody against both primary antibodies. SDS-polyacrylamide gel (12%) was used for MnSOD Western blot analysis. Equal protein loading was confirmed on immunoblots using rabbit anti-actin antibody (Sigma). Bands were visualized by chemiluminescence (Pierce). All immunoblots were obtained from at least three separate experiments. Quantification of band intensity for HIF-1α was determined using Image J (NIH).

AE activity gels. MnSOD activity was visualized by native PAGE, which is based on the inhibition by SOD of the in-gel reduction of nitroblue tetrazolium (NBT; ref. 18). Briefly, cells were washed with PBS (pH 7.4), and lysates were prepared in NP40 lysis buffer [150 mmol/L NaCl, 1% Nonidet-P40, and 50 mmol/L Tris buffer (pH 8.0)]. Proteins were quantified using the Bio-Rad protein assay. Equal amounts of protein from different samples were loaded onto a polyacrylamide gel (12% running gel with a 5% stacking gel). After electrophoresis, gels were stained with 2.43 mmol/L NBT for 20 min in the dark and then rinsed with distilled water, and 28 mmol/L riboflavin/28 mmol/L TEMED were added and illuminated under fluorescent light. For catalase and glutathione peroxidase GPx activity, 8% and 10% running gel were used. For catalase activity, gels were incubated with 0.003% H₂O₂ for 10 min and then stained with 2% ferric chloride–2% potassium ferricyanide solution (19). For GPx activity, gels were soaked in 1 mmol/L reduced glutathione for 30 min, incubated with 0.008% cumene hydroperoxide for 10 min, and then finally stained with 1% ferric chloride–1% potassium ferricyanide solution (19).

AE activity assays. SOD activity was also measured by the modified NBT method, as described previously (20). Briefly, SOD activity was determined spectrophotometrically at 560 nm by measuring the reduction of NBT. The ΔO₂⁻/mols generated from the xanthine and xanthine oxidase system reduces NBT. The reduction of NBT is competitively inhibited in the presence of SOD. The amount of protein that inhibits the reduction of NBT to 50% of maximum is defined as one unit of SOD activity. MnSOD activity was determined in the presence of 5 mmol/L sodium cyanide. CuZnSOD activity was calculated by subtracting MnSOD activity from total SOD activity.

Superoxide radical anion formation in cultured cells. Electron paramagnetic resonance (EPR) spin trapping with DMPO was used to detect O₂⁻•. This technique involves an addition reaction of a short-lived radical to a diamagnetic compound (spin trap) to form a more stable free radical product (spin adduct), which can be studied by EPR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped; the hyperfine couplings of the spin adduct are characteristic of the original trapped radical. In brief, cells were washed with PBS and incubated with 100 mmol/L DMPO in chelated PBS (pH 7.4; ref. 21) for 15 min. The cells were then transferred to a TM quartz flat cell, and EPR spectra were recorded.

Figure 1. Transient siRNA knockdown of MnSOD. A, Western blot analysis of whole-cell lysates showing MnSOD protein levels of immortalized nonmalignant breast cells MCF-10A and human breast adenocarcinoma MCF-7. B and C, MCF-7 cells were transfected with MnSOD siRNA and nontargeting siRNA (siNeg) at different times and concentrations. Whole-cell lysates were analyzed by Western blot for MnSOD expression using actin as a protein loading control. MnSOD protein expression was compared relative to untransfected (control) or nontargeting siRNA. D, MnSOD activities were determined in MCF-7 cells transfected with 300 pmol siRNA for 72 h by nondissociating electrophoresis (12%-gel) stained for SOD activity. All results are representative of at least three separate experiments.
peak heights are in arbitrary units. Microwave power of 40 mW, and modulation frequency of 100 kHz. The EPR spectrum was measured by a fluorometric assay using a fluorophore and compared with standard concentrations of H2O2 determined by fluorescence intensity of each sample. The pH was corrected for any changes in pH. Excitation and emission wavelengths of 323 and 400 nm, respectively. The pH was measured spectrofluorometrically by measuring the dimer formed at pH 7.4. The H2O2 was allowed to accumulate in the modified HBSS for 1 h. The released H2O2 was measured by a fluorometric assay using pHPA fluorescence assay. pHPA (pH 10.6) is normalized to the protein. The intensity of the DMPO-OH signal corresponds to the relative rate of O2 formation. The EPR peak height (normalized to the amount of protein) measured from cells transfected with siRNA against MnSOD is significantly different from nontargeting siRNA and control (i.e., untransfected cells; P < 0.01). D, extracellular H2O2 accumulation was determined by pHPA fluorescence assay (P > 0.05 relative to nontargeting siRNA). All results are representative of at least three separate experiments.

**Results**

MnSOD protein expression was suppressed by transient siRNA transfection. To determine whether MnSOD could affect the expression of HIF-1α under hypoxic conditions, we first manipulated MnSOD levels in human breast adenocarcinoma MCF-7 cells using specific RNA interference. The protein level of MnSOD in MCF-7 cells was observed to be lower than those of immortalized, nonmalignant MCF10A breast cells (Fig. 1A). In MCF-7 cells, MnSOD protein expression was found to be suppressed by siRNA in a time-dependent and concentration-dependent manner (Fig. 1B and C). siRNA against MnSOD showed suppression of protein within 24 hours after transfection with maximal decrease at 72 hours. Nontargeting siRNA transfected cells were similar to untransfected control (Fig. 1B and C, Neg). The transfection conditions of 300 pmol for 72 hours were selected for use in all subsequent experiments. The activity of MnSOD in cells transfected with siRNA was below the limit of detection of the spectroscopic-based assay. However, the suppression of the activity of MnSOD could be shown by nondissociating native gel electrophoresis (Fig. 1D). No changes in the activities of other AEs, such as CuZnSOD, catalase, or GPx, as measured by activity gels, were observed (data not shown).

**Inhibition of MnSOD by siRNA increased O2− levels and induced HIF-1α accumulation in cells exposed to 1% O2.** We
next determined the level of HIF-1α protein induction in MCF-7 cells after being transiently transfected with siRNA against MnSOD, nontargeting siRNA, or exposure to the transfection agent alone. The transfected cells were exposed to 1% O₂ for 4 h, and the relative levels of MnSOD protein were determined. After treatment with MnSOD siRNA relative to nontargeting siRNA or transfection reagent control, a decrease in MnSOD protein and a modest increase of HIF-1α protein was observed (Fig. 2A). This was further confirmed by the quantitation of the blot intensities. These results suggest that changes in MnSOD levels had an effect on HIF-1α accumulation under hypoxic conditions in MCF-7 cells.

To evaluate the functional consequences of MnSOD-mediated O₂⁻⁺ induction of HIF-1α, we determined whether inhibiting MnSOD actually results in an increased level of O₂⁻⁺, the substrate of MnSOD. MCF-7 cells were transfected with siRNA followed by exposure to 1% O₂. Free radical production was assessed by EPR by measuring the intensities of the DMPO-OH spin adduct, which corresponds to the relative rates of O₂⁻⁺ formation, which is inhibitable by SOD (not shown). After 4 hours of hypoxia, cells accumulated EPR-detectable DMPO-OH adducts (Fig. 2B). EPR spectra from cells transfected with MnSOD siRNA showed a greater peak height of the DMPO-OH spin adduct relative to nontargeting siRNA and untransfected controls. There were no significant differences in the intensities of the spectra observed from nontargeting siRNA and untransfected control. Quantitation of the spectral peak heights normalized to the amount of protein indicated a significant increase in accumulation of DMPO-OH measured from cells transfected with MnSOD siRNA relative to nontargeting siRNA (P < 0.01; Fig. 2C). Extracellular accumulation of H₂O₂ from MnSOD siRNA transfected cells showed no significant difference relative to nontargeting siRNA transfected cells (Fig. 2D). These results show that the suppression of MnSOD by siRNA significantly increased the level of O₂⁻⁺ and concomitantly increased HIF-1α induction under hypoxia in MCF-7 cells. The increase in accumulation of HIF-1α protein when MnSOD is decreased in this cell line suggests that O₂⁻⁺ plays a role in the regulation of HIF-1α.

**Scavenging of O₂⁻⁺ by the spin traps POBN and DMPO suppressed HIF-1α under 1% O₂ conditions.** By using siRNA, we showed that a decreased level of MnSOD resulted in a significant increase in the steady-state level of O₂⁻⁺ and an induction of HIF-1α protein. To determine whether O₂⁻⁺ is an important molecular species responsible for the induction of HIF-1α during hypoxia, levels of O₂⁻⁺ were lowered in MCF-7 cells with the spin trapping agents POBN or DMPO. Different concentrations of POBN or DMPO (33–100 mmol/L) were introduced to the cells under both 21% and 1% O₂ conditions, and HIF-1α protein was determined. These high concentrations are necessary because of their low rate of reaction with O₂⁻⁺ compared with the naturally occurring SODs. POBN was present through the 4-hour incubation. However, because of its high reactivity and propensity to form oxidation products, DMPO was added only for the final hour of the hypoxic incubation. As expected, HIF-1α protein was detectable only under 1% O₂. Cells treated with these spin traps had decreased levels of HIF-1α after the hypoxic incubation. HIF-1α protein induction seemed to be decreased in a spin trap concentration-dependent manner (Fig. 3A). To monitor other consequences of introducing spin trapping agents during the hypoxic incubation, the ability of cells to produce ROS after the hypoxic incubation was examined. Relative levels of ROS were assessed under both normoxia and hypoxia. Media was removed, and fresh DMPO (100 mmol/L) in chelated-PBS was introduced. The signal height from the EPR spectra normalized to the amount of protein was lower from those cells exposed to spin traps.

![Figure 3. Scavenging of O₂⁻⁺ by spin traps POBN or DMPO suppressed HIF-1α protein under 1% O₂ conditions.](cancerres.aacrjournals.org)
trap (100 mmol/L) during the hypoxic incubation (Fig. 3B). These results are consistent with O$_2^*$ being involved in HIF-1α induction under hypoxia.

Scavenging of O$_2^*$ by Tempol affected HIF-1α protein accumulation under 1% O$_2$ conditions. A. MCF-7 cells were treated with Tempol at different concentrations (0.1–40 mmol/L) at 1% O$_2$ for 4 h. Whole-cell lysates were analyzed for the expression of HIF-1α and MnSOD protein by Western blot analysis. B. EPR signal height of DMPO-OH normalized to the amount of protein obtained from cells treated with various concentrations of Tempol ($P < 0.05$ relative to 1% O$_2$ control). #, DMPO-OH adduct peaks in the presence of 10 to 40 mmol/L Tempol were masked by the Tempol EPR signal and thus could not be quantified. C. extracellular H$_2$O$_2$ formation was determined in Tempol-treated cells by the pHPA fluorescence assay ($P < 0.05$ relative to 1% control).

Figure 4. Scavenging of O$_2^*$ by Tempol affected HIF-1α protein accumulation under 1% O$_2$ conditions. A. MCF-7 cells were treated with Tempol at different concentrations (0.1–40 mmol/L) at 1% O$_2$ for 4 h. Whole-cell lysates were analyzed for the expression of HIF-1α and MnSOD protein by Western blot analysis. B. EPR signal height of DMPO-OH normalized to the amount of protein obtained from cells treated with various concentrations of Tempol ($P < 0.05$ relative to 1% O$_2$ control). #, DMPO-OH adduct peaks in the presence of 10 to 40 mmol/L Tempol were masked by the Tempol EPR signal and thus could not be quantified. C. extracellular H$_2$O$_2$ formation was determined in Tempol-treated cells by the pHPA fluorescence assay ($P < 0.05$ relative to 1% control).

Figure 5. SOD mimic suppressed HIF-1α expression under 1% O$_2$ conditions. A. Different concentrations (10–200 μmol/L) of AEOL10113 were added to MCF-7 cells at 21% O$_2$ for 4 h followed by incubation at 1% O$_2$ for 4 h. HIF-1α protein expression was analyzed from whole-cell lysates by Western blot. Relative band intensities for HIF-1α are presented under the blots. B. Quantified data from the EPR spectra normalized to the amount of protein obtained from cells treated with different concentrations of AEOL10113 ($P < 0.05$ relative to 1% control). C. extracellular H$_2$O$_2$ accumulation was determined by the pHPA fluorescence assay ($P < 0.05$ relative to 1% untreated control).
SOD mimic suppressed HIF-1α induction under 1% O2. To further analyze the effects of ROS removal and/or ROS generation in MCF-7 cells, we used the AEOL10113, a small molecular weight manganese-containing porphyrin that has potent SOD mimic activity (25). It has been reported that AEOL10113 can reduce hypoxia-induced O$_2^-$ levels and VEGF production by macrophages (26). Different concentrations of the compound were introduced to MCF-7 cells at 21% O$_2$ for 4 h, then cells were treated with hypoxia, and HIF-1α protein was determined. At a low concentration of AEOL10113 (10 μmol/L), HIF-α was found to be decreased; an additional decrease was seen with 20 μmol/L mimetic. However, when AEOL10113 concentrations were increased further, HIF-1α levels increased (Fig. 5A). The changes in the fold intensities were measured by integrated density values. Consistent with its SOD mimetic activity, the level of O$_2^-$ decreased (Fig. 5B) and the level of H$_2$O$_2$ increased (Fig. 5C; ref. 27). Interestingly, at 50 μmol/L, AEOL10113, a concentration wherein O$_2^-$ was lowest relative to untreated cells (P < 0.05), the accumulation of H$_2$O$_2$ was greatest. This biphasic response parallels to that observed by Wang et al. (16).

Changes in superoxide levels affected clonogenic survival of MCF-7 cells. In spin trapping and O$_2^-$ scavenger experiments, we observed a significant decrease in DMPO-OH signals, which implies that O$_2^-$ levels in the cells had been altered. We hypothesized that the changes in O$_2^-$ levels induced by these spin traps and O$_2^-$ scavengers may be harmful and thus contribute to cell mortality. To test the hypothesis, we evaluated the cytotoxicity of these agents by determining clonogenic survival of the cells after treatment during the exposure to hypoxia. Cells treated with either POBN or DMPO during 1% O$_2$ exposure showed no significant difference in survival fractions relative to untreated control cells (P > 0.05), but the surviving fractions were significantly decreased relative to untreated cells at 21% O$_2$ (P < 0.01; Fig. 6A and B). Cells treated with either 1% O$_2$ or 1% O$_2$ + Tempol showed a significant decrease in survival fraction (P < 0.01) relative to untreated cells at 21% O$_2$; cells treated with 1% O$_2$ + Tempol exposure showed a significant decrease in survival fraction relative to control 1% (P < 0.01) only when the concentrations of Tempol were higher than 10 mmol/L (Fig. 6C). However, cells treated with AEOL10113 at 1% O$_2$ conditions showed no significant difference in survival fraction compared with untreated cells at 21% and 1% O$_2$ only cells treated with 1% O$_2$ + AEOL10113 at 200 μmol/L exposure showed a significant decrease in survival fraction relative to 1% O$_2$ + AEOL10113 at 20 μmol/L (P < 0.05; Fig. 6D).

Discussion

In recent years, much effort has been devoted to the use of respiratory inhibitors or ϕ$^0$ cells to examine the role of the mitochondrial electron transport chain in the regulation of HIF-1α. It is likely that mechanisms of O$_2$ sensing and signaling during hypoxia are associated with mitochondrial ROS generation and involve different pathways in different cell types (10, 17, 28). Work using genetic approaches has shown that HEK293 cells transfected with siRNA against the Rieske iron-sulfur protein of mitochondria complex III failed to stabilize HIF-1α protein during hypoxia. In addition, both wild-type human fibroblasts and cells that had an impairment of oxidative phosphorylation exhibited an increase in HIF-1α protein stabilization when exposed to hypoxia (1.5% O$_2$), which was prevented by the addition of myxothiazol. The authors concluded that oxidative phosphorylation is not required for the hypoxic stabilization of HIF-1α but mitochondrial ROS are needed (17). Therefore, ROS play a major role in stabilizing HIF-1α.

The exact molecular nature of ROS responsible for regulation of HIF-1α under hypoxia is not clear. Wang et al. found that
overexpression of MnSOD resulted in a biphasic effect on HIF-1α protein levels (16). They showed that with relatively low overexpression of MnSOD, HIF-1α decreased. Because an increase in MnSOD would lower the steady-state level of superoxide, this observation suggests that superoxide may play a role in the stabilization of HIF-1α protein. However, when MnSOD was highly overexpressed, HIF-1α was again present. Because high levels of MnSOD can lead to greater fluxes of H2O2, this suggests that H2O2 may also regulate HIF-1α. Goyal et al. found that overexpression of a NADPH oxidase 1 (Nox1), which generates high fluxes of O2·−, in human lung adenocarcinoma A549 cells resulted in accumulation of HIF-1α in normoxia (29); under hypoxia (1% O2), an additional increase was observed. These effects could be reversed by the flavoprotein inhibitor diphenylene iodonium or by catalase. These observations are consistent with high levels of H2O2 being able to activate HIF-1α. Therefore, the appearance of HIF-1α at higher levels of MnSOD, as well as with the activation of Nox1, suggests that high levels of H2O2 can lead to accumulation of HIF-1α. To address these possible roles of ROS in stabilizing HIF-1α, we carried out experiments using ROS scavengers. When MCF-7 cells were exposed to nontoxic concentrations of Tempol (<10 mmol/L), the protein level of HIF-1α increased (Fig. 4A). Concomitantly, the level of O2·− as seen by the intensity of the DMPO-OH signal, was decreased (Fig. 4B). At nontoxic concentrations of Tempol, there was no change in the level of H2O2 (Fig. 4C); concentrations of Tempol (>1 mmol/L) are toxic, as seen by decreased cell survival (Fig. 6C). Tempol is a redox active compound and may well intercept the ferryl state of PHD, not allowing the hydroxylation of HIF-1α to occur. That Tempol allows HIF-1α to accumulate may explain the many positive in vivo observations reported with this compound (30). Thus, firm conclusions on the identity of a specific ROS that regulates HIF-1α cannot be made from these observations. To better probe for the identity of ROS, we carried out experiments with an SOD mimic, AEO10113 (24). This SOD mimic had no effect on cell survival (Fig. 6D).

To address better the role of ROS in the modulation of HIF-1α, we introduced varying levels of AEO10113 to cells in combination with exposure to hypoxia. Similar to the observations of Wang et al. (16), we observed a biphasic effect in the accumulation of HIF-1α with varying concentration of AEO10113. At low concentrations of SOD mimic, we observed decreased levels of HIF-1α in MCF-7 cells and a concomitant decrease in the levels of O2·− during hypoxia as studied by EPR spin trapping (Fig. 5A and B). Whereas at higher concentrations of SOD mimic, corresponding increases in H2O2 were seen with parallel increases in HIF-1α protein. Because the SOD mimic altered the levels of both O2·− and H2O2, the precise roles of O2·− and H2O2 cannot be deconvoluted. Therefore, we took another approach to alter the endogenous levels of O2·− and MnSOD. In the experiments with Tempol and AEO10113, the goal was to increase the effective SOD-like activity in cells. To specifically decrease the endogenous MnSOD activity, we used siRNA against MnSOD. This should result in an increase in the steady-state level of O2·−, which should lead to an increase in HIF-1α. Indeed, upon introduction of siRNA, we observed the anticipated lowering of MnSOD and an increase in both O2·− and HIF-1α (Fig. 2A and C). There was no detectable change in the level of H2O2 (Fig. 2D). These observations point directly to O2·− as a modulator of HIF-1α stabilization during hypoxia.

To provide additional evidence for the role of O2·− in modulating HIF-1α, we used the spin traps POBN and DMPO as scavengers of O2·−. Lowering the steady-state level of O2·− should lower HIF-1α under hypoxia. We found that both POBN and DMPO lowered the level of HIF-1α protein (Fig. 3A). Neither POBN nor DMPO were significantly toxic to the cells under our experimental conditions (Fig. 6A and B). Taking all observations together, it is clear that O2·− has a major role in regulating HIF-1α.

Here, we propose that MnSOD plays an important role in regulating HIF-1α accumulation during hypoxia by modulating the levels of O2·−. To our knowledge, this is the first demonstration that MnSOD regulates HIF-1α via O2·−. These results should provide a better understanding of the biological role of MnSOD in regulating HIF-1α in tumor cells.

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